

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 September 2001 (27.09.2001)

PCT

(10) International Publication Number
WO 01/71019 A1

(51) International Patent Classification⁷: **C12N 15/90**,
A01K 67/027, 67/033, A01H 1/00

(21) International Application Number: **PCT/EP01/03341**

(22) International Filing Date: **21 March 2001 (21.03.2001)**

(25) Filing Language: **English**

(26) Publication Language: **English**

(30) Priority Data:
0006753.8 21 March 2000 (21.03.2000) GB
60/195,678 7 April 2000 (07.04.2000) US

(71) Applicants and

(72) Inventors: **SAVAKIS, Charalambos** [GR/GR];
IMBB-Forth, P.O. Box 1527, GR-711 10 Heraklion
(GR). **GROSVELD, Frank** [NL/NL]; Maaik Weezenaar
Straat 27, NL-3065 GE Rotterdam (NL).

(74) Agents: **MASCHIO, Antonio** et al.; D Young & Co., 21
New Fetter Lane, London EC4A 1DA (GB).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK,
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,
MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,
TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: **METHOD OF GENERATING TRANSGENIC ORGANISMS USING TRANSPOSONS**

(57) Abstract: The invention comprises the crossing of two transgenic organisms, wherein one organism comprises, preferably as a result of transgenesis, one or more copies of a transposon, for example the Minos transposon; and the other organism comprises, preferably as a result of transgenesis, one or more copies of the cognate transposase, for example the Minos transposase.

BEST AVAILABLE COPY

WO 01/71019 A1

METHOD OF GENERATING TRANSGENIC ORGANISMS USING TRANSPOSONS

The present invention relates to transgenic organisms, and methods for producing such organisms. In particular, the invention relates to transgenic organisms which comprise
5 one or more insertions of a transposable element, or transposon. The transposon is preferably the Minos transposon.

Transposons are genetic elements which are capable of "jumping" or transposing from one position to another within the genome of a species. They are widely distributed
10 amongst animals, including insects.

The availability of genetic methodologies for functional genomic analysis is crucial for the study of gene function and genome organization of complex eukaryotes. Of the three "classical" model animals, the fly, the worm and the mouse, efficient transposon based
15 insertion methodologies have been developed for *D. melanogaster* and for *C. elegans*. The introduction of *P* element mediated transgenesis and insertional mutagenesis in *Drosophila* (Spradling & Rubin (1982) *Science* 218, 341-347) transformed *Drosophila* genetics and formed the paradigm for developing equivalent methodologies in other eukaryotes. However, the *P* element has a very restricted host range, and therefore other
20 elements have been employed in the past decade as vectors for gene transfer and/or mutagenesis in a variety of complex eukaryotes, including nematodes, plants, fish and a bird.

Minos is a transposable element derived from *Drosophila* (Franz and Savakis, (1991) NAR 19:6646). It is described in US patent 5,840,865, which is incorporated herein by
25 reference in its entirety. The use of *Minos* to transform insects is described in the foregoing US patent.

Mariner is a transposon originally isolated from *Drosophila*, but since discovered in
30 several invertebrate and vertebrate species. The use of *mariner* to transform organisms is described in International patent application WO99/09817.

Hermes is derived from the common housefly. Its use in creating transgenic insects is described in US patent 5,614,398, incorporated herein by reference in its entirety.

5 *PiggyBac* is a transposon derived from the baculovirus host *Trichplusia ni*. Its use for germ-line transformation of Medfly has been described by Handler *et al.*, (1998) PNAS (USA) 95:7520-5.

10 European Patent Application 0955364 (Savakis *et al.*, the disclosure of which is incorporated herein by reference) describes the use of Minos to transform cells, plants and animals. The generation of transgenic mice comprising one or more Minos insertions is described.

15 International Patent Application WO99/07871 describes the use of the Tc1 transposon from *C. elegans* for the transformation of *C. elegans* and a human cell line.

The use of *Drosophila* P-elements in *D. melanogaster* for enhancer trapping and gene tagging has been described; see Wilson *et al.*, (1989) Genes dev. 3:1301; Spradling *et al.*, (1999) Genetics 153:135.

20 In the techniques described in the prior art, the use of the cognate transposase for inducing transposon jumping is acknowledged to be necessary. Transgenic animals, where described, have the transposase provided in *cis* or *trans*, for example by cotransformation with transposase genes.

25 **Summary of the Invention**

We have now developed an improved protocol for the generation of transgenic animals using transposable elements as a genetic manipulation tool. In the improved protocol, the transposase function is provided by crossing of transgenic organisms in order to produce
30 organisms containing both transposon and transposase in the required cells or tissues. The invention allows tissue-specific, regulatable transposition events to be used for genetic manipulation of organisms.

According to a first aspect of the invention, there is provided a method for generating a transgenic organism, comprising the steps of:

(a) providing a first transgenic organism, which organism comprises, within at least a portion of its tissues or cells, one or more copies of a transposon;

5 (b) providing a second organism, which organism comprises, in the genome of at least a portion of its tissues or cells, a transposase or one or more copies of a gene encoding a transposase; and

(c) crossing the organism so as to obtain transgenic progeny which comprise, in at least a portion of their tissues or cells, both the transposon and the transposase.

10

The invention comprises the crossing of two transgenic organisms, wherein one organism comprises, preferably as a result of transgenesis, one or more copies of a transposon; and the other organism comprises, preferably as a result of transgenesis, one or more copies of the cognate transposase.

15

It is highly preferred that the transposon is Minos; and/or that the organism is a mammal.

20

A "cognate" transposase, as referred to herein, is any transposase which is effective to activate transposition of the transposon, including excision of the transposon from a first integration site and/or integration of the transposon at a second integration site. Preferably, the cognate transposase is the transposase, which is naturally associated with the transposon in its *in vivo* situation in nature. However, the invention also encompasses modified transposases, which may have advantageously improved activities within the scope of the invention.

25

The transposon may be a natural transposon. Preferably, it is a type-2 transposon, such as Minos. Most advantageously, it is Minos. Alternative transposons include mariner, Hermes and piggyBac.

30

The invention moreover relates to the use of modified transposons, which incorporate one or more heterologous coding sequences and/or expression control sequences. Such coding sequences may include selectable and/or unselectable marker genes, which permit the identification of transposons in the genome and cloning of the loci into which the

transposons have been integrated. Suitable markers include fluorescent and/or luminescent polypeptides, such as GFP and derivatives thereof, luciferase, β -galactosidase, or chloramphenicol acetyl transferase (CAT).

- 5 In an advantageous embodiment, the transposase may be expressed in the transgenic organisms in a regulatable manner. This means that the activation of the transposon can be determined according to any desired criteria. For example, the transposase may be placed under the control of tissue-specific sequences, such that it is only expressed at desired locations in the transgenic organism. Such sequences may, for example, comprise
10 tissue-specific promoters, enhancers and/or locus control sequences.

Moreover, the transposase may be placed under the control of one or more sequences which confer developmentally-regulated expression. This will result in the transposons being activated at a given stage in the development of the transgenic animal or its
15 progeny.

Using the techniques of the invention, gene modification events can be observed at a very high frequency, due to the efficiency of mobilisation and insertion of transposons. Moreover, the locus of the modification may be identified precisely by locating the
20 transposon insertion. Sequencing of flanking regions allows identification of the locus in databases, potentially without the need to sequence the locus. Moreover, the use of transposons provides a reversible mutagenesis strategy, such that modifications can be reversed in a controlled manner.

- 25 The transposon may be inserted into a gene. Preferably, the transposon is inserted into a highly transcribed gene, resulting in the localisation of said transposon in open chromatin. This increases the accessibility of the transposon which may result in increased transposition frequencies.

- 30 Moreover, the transposon may itself comprise, between the transposon ends, a highly-transcribed gene. This will cause activation of the chromatin structure into which the transposon integrates, facilitating access of the transposase thereto.

The transposon may be inserted into the gene by recombination. Furthermore, the transposon may be inserted into the gene by recombination in cells such as ES cells.

According to a second aspect of the invention, there is provided a method for detecting and characterising a genetic mutation in a transgenic organism, comprising the steps of:

(a) generating a transgenic organism by a procedure according to the first aspect of the invention;

(b) characterising the phenotype of the transgenic organism;

(c) detecting the position of one or more transposon insertion events in the genome of the organism; and

(d) correlating the position of the insertion events with the observed phenotype, the position of the insertion events being indicative of the location of one or more gene loci connected with the observed phenotype.

The generation of genetic mutations in transgenic organisms as a result of transposon insertion after crossing of transgenic organisms according to the invention gives rise to novel phenotypic variations in the organisms, which can be traced back to insertion events in the genome of the organism. Transposon excisions characteristically result in the insertion of a small number of nucleotides into the host genome, left behind by the transposon and the recombination events associated with its insertion and subsequent excision. Small insertions may have small phenotypic effects, for example resulting from the insertion of a few amino acids into the sequence of a polypeptide. Alternatively, the effects may be more pronounced, possibly including the complete inactivation of a gene.

Transposon insertions are more likely to have significant phenotypic consequences, on the grounds that the insertion is much larger.

If a transposon is inserted into an intron of a gene, resulting in inactivation of the gene, its excision leads, in the majority of cases, to restoration of gene activity. Thus, the invention provides a reversible mutagenesis procedure, in which a gene can be inactivated and subsequently restored.

Insertion events may be detected by screening for the presence of the transposon, by probing for the nucleic acid sequence of the transposon. Excisions may also be identified by the "signature" sequence left behind upon excision.

- 5 In a preferred embodiment, transposons may be used to upregulate the expression of genes. For example, a transposon may be modified to include an enhancer or other transcriptional activation element. Mobilisation and insertion of such a transposon in the vicinity of a gene upregulates expression of the gene or gene locus. This embodiment has particular advantage in the isolation of oncogenes, which may be identified in clonal
10 tumours by localisation of the transposon.

According to a third aspect, there is provided a method for isolating a gene which is correlated with a phenotypic characteristic in a transgenic animal, comprising the steps of:

- 15 (a) generating a transgenic organism by a procedure according to the first aspect of the invention;
(b) characterising the phenotype of the transgenic organism;
(c) detecting the position of one or more transposon insertion events in the genome of the organism; and
20 (d) cloning the genetic loci comprising the insertions.

The invention provides clear advantages in functional genomics, since gene disruption or activation by transposon jumping is easily traced due to tagging by the transposon.

- 25 According to a fourth aspect, there is provided a method for isolating an enhancer in a transgenic animal, comprising the steps of:

- (a) generating a transgenic organism by a procedure according to the first aspect of the invention, wherein the transposon comprises a reporter gene under the control of a minimal promoter such that it is expressed at a basal level;
30 (b) assessing the level of expression of the reporter gene in one or more tissues of the transgenic organism;
(c) identifying and cloning genetic loci in which the modulation of the reporter gene is increased or decreased compared to the basal expression level; and

(d) characterising the cloned genetic loci.

According to a fifth aspect, there is provided a method for isolating an exon of an endogenous gene in a transgenic animal, comprising the steps of:

- 5 (a) generating a transgenic organism by a procedure according to the first aspect of the invention, wherein the transposon comprises a reporter gene which lack translation initiation sequences but includes splice acceptor sequences;
- (b) identifying tissues of the organism in which the reporter gene is expressed;
- (c) cloning the genetic loci comprising the expressed reporter gene.

10

The invention may be used to provided *in vivo* enhancer trap and exon trap functions, by inserting transposons which comprise marker genes which are modulated in their expression levels by the proximity with enhancers or exons. Suitable constructs for such applications are described in EP 0955364 and known in the art. Since transposon

15 activation may be effected in a tissue-specific or developmentally regulated manner, the invention permits the trapping of enhancers and/or exons which are subject to similar regulation in the transgenic organism.

According to a sixth aspect, there is provided a method for modulating the expression of a gene in an organism, comprising the steps of:

20

- (a) generating a library of transgenic organisms according to the first aspect of the invention;
- (b) selecting from said library one or more transgenic organisms in which the expression of a gene of interest is modulated as a result of one or more transposon
- 25 insertion events.

The invention moreover comprises transgenic animals suitable for crossing in a method according to the invention, and thus encompasses a transgenic organism comprising one or more copies of a heterologous transposon, said transgenic organism being free of

30 nucleic acid sequences encoding the cognate transposase enzyme, and a transgenic organism encoding a transposase enzyme, said transgenic organism being free of the cognate transposon.

Description of the Figures

- Figure 1.** *Minos* derived vectors. *Minos* inverted terminal repeats are shown as thick black arrows. White blocks outside these arrows indicate the sequences flanking the original *Minos* element in the *D. hydei* genome. Arrowheads indicate the positions of primers used to detect *Minos* excisions. Small arrows indicate the direction of transcription of the GFP and transposase genes. Black bars represent fragments used as probes.
- Figure 2.** Tissue specific expression of *Minos* transposase in transgenic mice. Northern blot analysis of thymus, spleen and kidney RNA isolated from TM2/+ mice (40-hr exposure). Control RNA is from thymus of a non-transgenic mouse. The lower panel shows the signal obtained upon re-hybridisation of the same filter with a mouse actin probe (3-hr exposure).
- Figure 3.** Transposase dependent, tissue-specific excision of a *Minos* transposon in mice. Oligonucleotide primers flanking the transposon were used for PCR and the products were analysed by agarose gel electrophoresis. Left panel: Transposase-dependent excision in the thymus. Template DNA used: Lane 1, non transgenic; lane 2, TM2/+; lanes 3-7, MCG/+; lanes 8-12, MCG/+ TM2/+. Right panel: Excision in various tissues of transposase-expressing mice. Template DNA used: Lanes 1, 3, 5, 7, 9, 11, from MCG/+ mice. Lanes 2, 4, 6, 8, 10, 12, from MCG/+ TM2/+ mice. Lanes 1-2, thymus. Lanes 3-4, spleen. Lanes 5-6, liver. Lanes 7-8, kidney. Lanes 9-10, brain. Lanes 11-12, muscle. Lane 13, no DNA added.
- Figure 4.** Footprints left behind at chromosomal sites after *Minos* excision. DNA is extracted from thymus and spleen of a double transgenic mouse (top), or from an embryonic fibroblast cell line from a MCG/+ mouse after transfection with a transposase-expressing plasmid (bottom) and used as template for PCR with the flanking primers. PCR-amplified bands were cloned and 32 clones (19 from thymus and spleen and 13 from fibroblast cells) were sequenced. TA is the target site duplication. Nucleotides in red correspond to the ends of the transposon terminal repeats; nucleotides in blue are of unknown origin. The flanking nucleotides and TA repeats are aligned.

Figure 5. FISH analysis of *Minos* transpositions in thymus and spleen. Chromosomes were stained with DAPI. Panels A and B are from the same MCG/+ metaphase nucleus, probed with a GFP and a telomere 14 specific probe, respectively. Panels C to F are nuclei probed with GFP. Panels C and D are from thymus and spleen respectively from the same MCG/+,TM2/+ mouse. Panels E and F are from spleen of two different MCG/+,TM2/+ mice. Yellow arrowheads indicate the original integration site of the transposon transgene, near the telomere of chromosome 14. Green arrowheads indicate the telomeres of chromosome 14. Red arrowheads indicate transposition events.

Detailed Description of the Invention

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.

A transgenic organism of the invention is preferably a multicellular eukaryotic organism, such as an animal, a plant or a fungus.

The organism is preferably an animal, more preferably a mammal. Advantageously, the organism is not an insect. Preferably, the organism is not *D. melanogaster*.

In a preferred embodiment, the organism is a plant.

Animals include animals of the phyla cnidaria, ctenophora, platyhelminthes, nematoda, annelida, mollusca, chelicerata, uniramia, crustacea and chordata. Uniramians include the subphylum hexapoda that includes insects such as the winged insects. Chordates includes vertebrate groups such as mammals, birds, reptiles and amphibians. Particular examples of mammals include non-human primates, cats, dogs, ungulates such as cows, goats, pigs, sheep and horses and rodents such as mice, rats, gerbils and hamsters.

Plants include the seed-bearing plants angiosperms and conifers. Angiosperms include dicotyledons and monocotyledons. Examples of dicotyledonous plants include tobacco, (*Nicotiana plumbaginifolia* and *Nicotiana tabacum*), arabidopsis (*Arabidopsis thaliana*), *Brassica napus*, *Brassica nigra*, *Datura innoxia*, *Vicia narbonensis*, *Vicia faba*, pea
5 (*Pisum sativum*), cauliflower, carnation and lentil (*Lens culinaris*). Examples of monocotyledonous plants include cereals such as wheat, barley, oats and maize.

Production of transgenic animals

- 10 Techniques for producing transgenic animals are well known in the art. A useful general textbook on this subject is Houdebine, Transgenic animals – Generation and Use (Harwood Academic, 1997) – an extensive review of the techniques used to generate transgenic animals from fish to mice and cows.
- 15 Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into, for example, fertilised mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into
20 a transgenic animal. In a highly preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals. Those
25 techniques as well known. See reviews of standard laboratory procedures for microinjection of heterologous DNAs into mammalian fertilised ova, including Hogan *et al.*, Manipulating the Mouse Embryo, (Cold Spring Harbor Press 1986); Krimpenfort *et al.*, Bio/Technology 9:844 (1991); Palmiter *et al.*, Cell, 41: 343 (1985); Kraemer *et al.*, Genetic manipulation of the Mammalian Embryo, (Cold Spring Harbor Laboratory Press
30 1985); Hammer *et al.*, Nature, 315: 680 (1985); Wagner *et al.*, U.S. Pat. No. 5,175,385; Krimpenfort *et al.*, U.S. Pat. No. 5,175,384, the respective contents of which are incorporated herein by reference

Another method used to produce a transgenic animal involves microinjecting a nucleic acid into pro-nuclear stage eggs by standard methods. Injected eggs are then cultured before transfer into the oviducts of pseudopregnant recipients.

- 5 Transgenic animals may also be produced by nuclear transfer technology as described in Schnieke, A.E. *et al.*, 1997, Science, 278: 2130 and Cibelli, J.B. *et al.*, 1998, Science, 280: 1256. Using this method, fibroblasts from donor animals are stably transfected with a plasmid incorporating the coding sequences for a polypeptide of interest under the control of regulatory sequences. Stable transfectants are then fused to enucleated oocytes,
10 cultured and transferred into female recipients.

Analysis of animals which may contain transgenic sequences would typically be performed by either PCR or Southern blot analysis following standard methods.

- 15 By way of a specific example for the construction of transgenic mammals, such as cows, nucleotide constructs comprising a sequence encoding a DNA binding molecule are microinjected using, for example, the technique described in U.S. Pat. No. 4,873,191, into oocytes which are obtained from ovaries freshly removed from the mammal. The oocytes are aspirated from the follicles and allowed to settle before fertilisation with thawed
20 frozen sperm capacitated with heparin and prefractionated by Percoll gradient to isolate the motile fraction.

- The fertilised oocytes are centrifuged, for example, for eight minutes at 15,000 g to visualise the pronuclei for injection and then cultured from the zygote to morula or
25 blastocyst stage in oviduct tissue-conditioned medium. This medium is prepared by using luminal tissues scraped from oviducts and diluted in culture medium. The zygotes must be placed in the culture medium within two hours following microinjection.

- Oestrous is then synchronized in the intended recipient mammals, such as cattle, by
30 administering coprostanol. Oestrous is produced within two days and the embryos are transferred to the recipients 5-7 days after oestrous. Successful transfer can be evaluated in the offspring by Southern blot.

Alternatively, the desired constructs can be introduced into embryonic stem cells (ES cells) and the cells cultured to ensure modification by the transgene. The modified cells are then injected into the blastula embryonic stage and the blastulas replaced into pseudopregnant hosts. The resulting offspring are chimeric with respect to the ES and host cells, and nonchimeric strains which exclusively comprise the ES progeny can be obtained using conventional cross-breeding. This technique is described, for example, in WO91/10741.

Production of transgenic plants

10

Techniques for producing transgenic plants are well known in the art. Typically, either whole plants, cells or protoplasts may be transformed with a suitable nucleic acid construct encoding a DNA binding molecule or target DNA (see above for examples of nucleic acid constructs). There are many methods for introducing transforming DNA constructs into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods include *Agrobacterium* infection (see, among others, Turpen *et al.*, 1993, J. Virol. Methods, 42: 227-239) or direct delivery of DNA such as, for example, by PEG-mediated transformation, by electroporation or by acceleration of DNA coated particles. Acceleration methods are generally preferred and include, for example, microprojectile bombardment. A typical protocol for producing transgenic plants (in particular monocotyledons), taken from U.S. Patent No. 5, 874, 265, is described below.

20

An example of a method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, non-biological particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

25

A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming both dicotyledons and monocotyledons, is that neither the isolation of protoplasts nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as a

30

stainless steel or Nyltex screen, onto a filter surface covered with plant cells cultured in suspension. The screen disperses the tungsten-DNA particles so that they are not delivered to the recipient cells in large aggregates. It is believed that without a screen intervening between the projectile apparatus and the cells to be bombarded, the projectiles
5 aggregate and may be too large for attaining a high frequency of transformation. This may be due to damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters. Filters containing the cells to be bombarded are positioned at an appropriate distance below the
10 microprojectile stopping plate. If desired, one or more screens are also positioned between the gun and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more clusters of cells transiently expressing a marker gene ("foci") on the bombarded filter. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and
15 average 2 to 3.

After effecting delivery of exogenous DNA to recipient cells by any of the methods discussed above, a preferred step is to identify the transformed cells for further culturing and plant regeneration. This step may include assaying cultures directly for a screenable
20 trait or by exposing the bombarded cultures to a selective agent or agents.

An example of a screenable marker trait is the red pigment produced under the control of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage, incubating the cells
25 at, e.g., 18°C and greater than $180 \mu\text{E m}^{-2} \text{ s}^{-1}$, and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media.

An exemplary embodiment of methods for identifying transformed cells involves
30 exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

To use the bar-bialaphos selective system, bombarded cells on filters are resuspended in nonselective liquid medium, cultured (e.g. for one to two weeks) and transferred to filters overlaying solid medium containing from 1-3 mg/l bialaphos. While ranges of 1-3 mg/l will typically be preferred, it is proposed that ranges of 0.1-50 mg/l will find utility in the practice of the invention. The type of filter for use in bombardment is not believed to be particularly crucial, and can comprise any solid, porous, inert support.

Cells that survive the exposure to the selective agent may be cultured in media that supports regeneration of plants. Tissue is maintained on a basic media with hormones for about 2-4 weeks, then transferred to media with no hormones. After 2-4 weeks, shoot development will signal the time to transfer to another media.

Regeneration typically requires a progression of media whose composition has been modified to provide the appropriate nutrients and hormonal signals during sequential developmental stages from the transformed callus to the more mature plant. Developing plantlets are transferred to soil, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO₂, and 250 $\mu\text{E m}^{-2} \text{ s}^{-1}$ of light. Plants are preferably matured either in a growth chamber or greenhouse. Regeneration will typically take about 3-12 weeks. During regeneration, cells are grown on solid media in tissue culture vessels. An illustrative embodiment of such a vessel is a petri dish. Regenerating plants are preferably grown at about 19°C to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

Genomic DNA may be isolated from callus cell lines and plants to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art such as PCR and/or Southern blotting.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found

in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

The vector system used may comprise one vector, but it can comprise at least two vectors.

- 5 In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

- 10 One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* (An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208).

- 15 Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

Transposons

- 20 Minos transposons, and their cognate transposase, are described in detail in US patent 5,840,865 and European patent application EP 0955364, the disclosures of which are incorporated herein by reference. Minos transposons may be modified, for instance to insert one or more selectable marker genes for example as referred to herein, according to general techniques. Specific techniques for modifying Minos are set forth in EP
25 0955364.

Marker genes

- 30 Preferred marker genes include genes which encode fluorescent polypeptides. For example, green fluorescent proteins ("GFPs") of cnidarians, which act as their energy-transfer acceptors in bioluminescence, can be used in the invention. A green fluorescent protein, as used herein, is a protein that fluoresces green light, and a blue fluorescent protein is a protein that fluoresces blue light. GFPs have been isolated from the Pacific

Northwest jellyfish, *Aequorea victoria*, from the sea pansy, *Renilla reniformis*, and from *Phialidium gregarium*. (Ward *et al.*, 1982, Photochem. Photobiol., 35: 803-808; Levine *et al.*, 1982, Comp. Biochem. Physiol., 72B: 77-85). See also Matz, *et al.*, 1999, *ibid* for fluorescent proteins isolated recently from Anthoza species (accession nos. AF168419, AF168420, AF168421, AF168422, AF168423 and AF168424).

A variety of *Aequorea*-related GFPs having useful excitation and emission spectra have been engineered by modifying the amino acid sequence of a naturally occurring GFP from *Aequorea victoria* (Prasher *et al.*, 1992, Gene, 111: 229-233; Heim *et al.*, 1994, Proc. Natl. Acad. Sci. U.S.A., 91: 12501-12504; PCT/US95/14692). As used herein, a fluorescent protein is an *Aequorea*-related fluorescent protein if any contiguous sequence of 150 amino acids of the fluorescent protein has at least 85% sequence identity with an amino acid sequence, either contiguous or non-contiguous, from the wild-type *Aequorea* green fluorescent protein (SwissProt Accession No. P42212). More preferably, a fluorescent protein is an *Aequorea*-related fluorescent protein if any contiguous sequence of 200 amino acids of the fluorescent protein has at least 95% sequence identity with an amino acid sequence, either contiguous or non-contiguous, from the wild type *Aequorea* green fluorescent protein of SwissProt Accession No. P42212. Similarly, the fluorescent protein may be related to *Renilla* or *Phialidium* wild-type fluorescent proteins using the same standards.

Aequorea-related fluorescent proteins include, for example, wild-type (native) *Aequorea victoria* GFP, whose nucleotide and deduced amino acid sequences are presented in Genbank Accession Nos. L29345, M62654, M62653 and others *Aequorea*-related engineered versions of Green Fluorescent Protein, of which some are listed above. Several of these, i.e., P4, P4-3, W7 and W2 fluoresce at a distinctly shorter wavelength than wild type.

Identification of insertion and excision events

30

Minos transposons, and sites from which transposons have been excised, may be identified by sequence analysis. Minos typically integrates at a TA base pair, and on excision leaves behind a duplication of the target TA sequence, flanking the four terminal

nucleotides of the transposon. The presence of this sequence, or related sequences, may be detected by techniques such as sequencing, PCR and/or hybridisation.

5 Inserted transposons may be identified by similar techniques, for example using PCR primers complementary to the terminal repeat sequences.

Regulation of transposase expression

10 Coding sequences encoding the transposase may be operatively linked to regulatory sequences which modulate transposase expression as desired. Control sequences operably linked to sequences encoding the transposase include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host organism in which the expression of the transposase is required. The term promoter is well-known in the art and encompasses nucleic acid regions ranging in size
15 and complexity from minimal promoters to promoters including upstream elements and enhancers.

The promoter is typically selected from promoters which are functional in cell types homologous to the organism in question, or the genus, family, order, kingdom or other
20 classification to which that organism belongs, although heterologous promoters may function – eg. some prokaryotic promoters are functional in eukaryotic cells. The promoter may be derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a
25 ubiquitous manner (such as promoters of α -actin, β -actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). They may also be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus
30 (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

It is moreover advantageous for the promoters to be inducible so that the levels of expression of the transposase can be regulated. Inducible means that the levels of

expression obtained using the promoter can be regulated. A widely used system of this kind in mammalian cells is the tetO promoter-operator, combined with the tetracycline/doxycycline-repressible transcriptional activator tTA, also called Tet-Off gene expression system (Gossen, M. & Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline responsive promoters. Proc. Natl. Acad. Sci. USA 89:5547-5551), or the doxycycline-inducible rtTA transcriptional activator, also called Tet-On system (Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. & Bujard, H. (1995) Transcriptional activation by tetracycline in mammalian cells. Science 268:1766-1769).

10

In the Tet-Off system, gene expression is turned on when tetracycline (Tc) or doxycycline (Dox; a Tc derivative) is removed from the culture medium. In contrast, expression is turned on in the Tet-On system by the addition of Dox. Procedures for establishing cell lines carrying the transcriptional activator gene and the Tet-regulatable gene stably integrated in its chromosomes have been described. For example see <http://www.clontech.com/techinfo/manuals/PDF/PT3001-1.pdf>. For example, the Tet-On system may be employed for tetracycline-inducible expression of Minos transposase in a transgenic animal. A doubly transgenic animal is generated by standard homologous recombination ES cell technology. Two constructs are used: first, a construct containing the rtTA gene under a constitutive promoter. An example of such construct is the pTet-On plasmid (Clontech) which contains the gene encoding the rtTA activator under control of the Cytomegalovirus immediate early (CMV) promoter. The rtTA transcriptional activator encoded by this construct is active only in the presence of Doxycycline. The second construct contains the Minos transposase gene under control of the tetracycline-response element, or TRE. The TRE consists of seven direct repeats of a 42-bp sequence containing the tet operator (tetO), and is located just upstream of the minimal CMV promoter, which lacks the enhancer elements normally associated with the CMV immediate early promoter. Because these enhancer elements are missing, there is no "leaky" expression of transposase from the TRE in the absence of binding by rtTA. An example of such construct is the pTRE2 plasmid (Clontech) in the MCS of which is inserted the gene encoding Minos transposase. In cells stably transformed with the two constructs, rtTA is expressed but does not activate transcription of Minos transposase unless Doxycycline is administered to the animal.

Alternative inducible systems include or tamoxifen inducible transposase [a modified oestrogen receptor domain (Indra et al., Nucl Acid Res. 27, 4324-27, 1999) coupled to the transposase which retains it in the cytoplasm until tamoxifen is given to the culture], or a
5 RU418 inducible transposase (operating under the same principle with the glucocorticoid receptor; see Tsujita et al., J. Neuroscience, 19, 10318-23, 1999).

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used
10 comprising sequence elements from two or more different promoters described above.

The use of locus control regions (LCRs) is particularly preferred. LCRs are capable of conferring tightly-regulated tissue specific control on transgenes, and to greatly increase the fidelity of transgene expression. A number of LCRs are known in the art. These
15 include the β -globin LCR (Grosveld *et al.*, (1987) Cell 51:975-985); α -globin (Hatton *et al.*, (1990) Blood 76:221-227; and CD2 (Festenstein *et al.*, (1996) Science 271:1123-1125), plus immunoglobulins, muscle tissue, and the like.

Regulation of transposase and/or transposon expression may also be achieved through the
20 use of ES cells. Using transformed ES cells to construct chimeric embryos, it is possible to produce transgenic organisms which contain the transposase genes or transposon element in only certain of their tissues. This can provide a further level of regulation.

The regulation of expression of transposase may induce excision of a transposon. This
25 may be used to genetically manipulate an organism. As used herein, the term "genetically manipulate" refers to the manipulation of genes in an organism's genome and may include the insertion or excision of a gene or part of a gene.

The sequence of the transposase may be modified to optimise codon usage and thus,
30 increase transposition frequencies. Optimisation of codon usage is a method well known in the art to increase the expression levels of a given gene.

The invention is further described, for the purpose of illustration, in the following examples.

Examples

5

Plasmid constructions

The helper plasmid CD2/ILMi is constructed by subcloning the transposase cDNA (Klinakis et al. (2000) *EMBO reports*.1, 416-421) as an *Xba*I-blunt fragment into the
10 vector SVA(-). The SVA(-) vector is a derivative of the VA vector (Zhumabekov et al. (1995). *J Immunol Methods* 185, 133-140) with extended multiple cloning sites.

Transposon MiCMVGFP is constructed as follows: The plasmid pMILRTetR (Klinakis et al. (2000) *Ins. Mol. Biol.* 9, 269-275 (2000b) is cut with *Bam*HI and re-ligated to remove
15 the tetracycline resistance gene between the *Minos* ends, resulting in plasmid pMILR Δ BamHI. An *Asp*718/*Sac*I fragment from pMILR Δ BamHI, containing the *Minos* inverted repeats and original flanking sequences from *D. hydei*, is cloned into plasmid pPolyIII-I-lox (created by insertion of the *loxP* oligo:

20 ATAACCTTCGTATAGCATACATTATACGAAGTTAT

into the *Asp*718 site of the vector pPolyIII-I (accession No. M18131), resulting in plasmid ppolyMILR Δ BamHI. The final construct (pMiCMVGFP, figure 1) used for the generation of transgenic mice, is created by inserting into the *Spe* I site of
25 ppolyMILR Δ BamHI the 2.2 kb *Spe*I fragment from plasmid pBluescriptGFP, containing a humanised GFP gene (from Clontech plasmid pHGFP-S65T) driven by the CMV promoter and followed by the SV40 intervening sequence and polyadenylation signal.

Plasmid pJGD/ILMi (figure 1) is constructed as follows: A 1 kb *Eco*RV/*Not*I fragment
30 containing the *Minos* transposase cDNA is cloned into *Eco*RV/*Not*I of plasmid pJG-3 (the puro variant of pJG-1; Drabek et al. (1997) *Gene Ther.* 4, 93-100 (1997). The resulting plasmid (pJGD/transposase) that carries a CMV promoter upstream of the transposase cDNA, an intron with splice site and polyA from the human β globin gene and the

puromycin resistance gene driven by PGK promoter and followed by the poly(A) signal from the bovine growth hormone gene is used as the transposase source in transfections of embryonic fibroblasts.

5 Generation of transgenic mice.

The transposase-expressing TM2 mouse line is generated by injecting the 12.5 kb *Syl* fragment from the CD2/ILMi plasmid (figure 1) into CBA x C57 B1/10 fertilized oocytes. Transgenic founder animals are identified by Southern blotting of DNA from tail biopsies, using the 1 kb transposase cDNA fragment as a probe and crossed with F1 CBA x C57 B1/10 mice to generate lines.

The transposon-carrying MCG line is constructed by injecting the 3.2 kb *XhoI* fragment from the pMiCMVGFP plasmid into FVB X FVB fertilized oocytes. Transgenic founder animals are identified by Southern blotting of DNA from tail biopsies, using GFP DNA as a probe.

Cell culture, transfection

13.5 day pregnant females (from crosses between MCG heterozygous transgenic male and wt females) are sacrificed, embryos are isolated and part of the material is used for genotyping. The remaining embryonic tissue is minced using a pair of scissors and immersed in a thin layer of F10/DMEM culture medium supplemented with 10 % FCS and antibiotics. Two spontaneously immortalized mouse embryonic fibroblasts lines (MEFs) with MCG/+ genotype are obtained by subculturing of primary MEFs. They are stably transfected with 20µg of plasmid pJGD/ILMi linearised with *ScaI*, using Lipofectin (GibcoBRL). Transfectants are selected on puromycin at a concentration of 1 µg/ml.

Northern blot hybridisation

15 µg of total RNA isolated (Chomczynski & Sacchi (1987). *Analytical Biochem.* 162, 156-159) from kidney, thymus and spleen is subjected to electrophoresis in a 1.2% agarose gel containing 15% formaldehyde. Northern blot analysis is performed as

described previously (Sambrook *et al.* (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)

PCR analysis

5

Genomic DNA from different tissues is isolated with the DNeasy Tissue-Kit (QIAGEN) according to the manufacturers instructions. PCR reactions are performed using primers 11DML:

10 (5'AAGTGTAAGTGCTTGAAATGC-3')

and GOUN67:

(5'-GCATCAAATTGAGTTTTGCTC-3').

15

PCR conditions are as follows: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin; 1.2 units Taq 2000TM DNA Polymerase (STRATAGENE), 200 g template DNA and 10 pmol of each primer per 25 µl reaction. 43 or 60 cycles of 30'' at 94°C, 30'' at 59°C and 30'' at 72°C were performed. PCR products are cloned into the

20 PCR II TA cloning vector (Invitrogen) and are sequenced using the T7 primer.

DNA fluorescent *in situ* hybridisation (FISH) analysis

Cells from minced thymus or spleen are cultured for 48h in RPMI medium (GIBCO BRL) supplemented with 9% FCS (GIBCO BRL), 13.6% Hybridoma medium (GIBCO BRL), 3.4 µg/ml Lithium chloride (MERCK), 7.2 µg/ml Concanavaline-A (SIGMA), 22.7 i.u./ml Heparine (LEO), 50 µM Mercaptoethanol, 25.4 µg/ml L.P.S. (SIGMA), 10 ng/ml interleukin 6 (PEPROTECH EC LTD). Chromosome preparations and FISH are carried out as described previously (Mulder *et al.* (1995) *Hum Genet* 96, 133-141. The

30 737 bp *SacI/NotI* GFP fragment from the pMiCMVGFP construct is used as a probe. The probe is labelled with Biotin (Boehringer Mannheim) and immunochemically detected with FITC. A telomeric probe for chromosome 14 (Shi, Y. *et al.* (1997) *Genomics* 45,

42-47) is labelled with dioxygenin (Boehringer Mannheim) and immunochemically detected with Texas Red.

Example 1: Activation of Minos *in vivo* in a tissue-specific manner

5

Two transgenic mouse lines are generated to determine whether *Minos* can transpose in mouse tissues: One containing a *Minos* transposon and another containing the *Minos* transposase gene expressed in a tissue-specific manner. The transposon-carrying line (line MCG) contains a tandem array of a fragment containing a *Minos* transposon (MiCMVGFP, figure 1) containing the GFP gene under the control of the cytomagalovirus promoter. The transposon is engineered such that almost all sequence internal to the inverted repeats is replaced by the CMV/GFP cassette. Not containing the transposase-encoding gene, this transposon is non-autonomous, and can only be mobilized when a source of transposase is present. The transposase-expressing line (line 10 TM2) contains a tandem array of a construct comprising the *Minos* transposase cDNA under the control of the human CD2 locus, consisting of the CD2 promoter and LCR elements (pCD2/ILMi, figure 1). In transgenic mice, the human CD2 locus is transcribed at high levels in virtually all thymocytes as well as peripheral T cells (Zhumabekov, T. *et al.* (1995) *J Immunol Methods* 185, 133-140.

20

Heterozygous TM2/+ mice are tested for tissue-specific production of *Minos* transposase RNA by Northern blot analysis. *Minos* transposase mRNA is detected in thymus and spleen, the two organs with large numbers of T cells, but is not detected in other organs such as kidney (figure 2).

25

A PCR assay for transposon excision is used to detect active transposition by *Minos* transposase in mouse tissues, using primers that hybridise to the non-mobile *Drosophila hydei* sequences which flank the *Minos* transposon in the constructs shown in figure 1 (Klinakis *et al.* (2000) *Ins. Mol. Biol.* 9, 269-275). In *Drosophila* cells, transposase-mediated excision of *Minos* is followed by repair of the chromatid which usually leaves a characteristic 6-base pair footprint (Arca *et al.* (1997) *Genetics* 145, 267-279). With the specific pair of primers used in the PCR assay this creates a diagnostic 167 bp PCR 30 fragment (Catteruccia F. *et al.* (2000) *Proc. Natl. Acad. Sci. U S A* 97, 2157-2162). As

shown in figure 3, the diagnostic band is present in tissues of double transgenic (MCG/+ TM2/+) mice expressing the transposase, but not of MCG/+ mice, not expressing transposase. The identity of the fragment is confirmed by Southern blot analysis using a labelled DNA probe specific for the amplified sequence (data not shown). Excision is
5 detectable mainly in thymus and spleen of the double transgenics; lower levels of excision are detectable in liver (figure 3). Very low levels of excision can also be detected in kidney, brain, and skeletal muscle, after 15 additional cycles of amplification (data not shown). Low levels of expression of the human CD2 locus in liver and lung of transgenic mice has been documented previously (Lang, G. *et al.* (1988) *EMBO J.* 6,
10 1675-1682). We therefore attribute the excision detected in tissues other than thymus and spleen to the presence of small numbers of T cells or to the expression of transposase in non-T cells of these tissues due to position effects.

Example 2: Detection of transposition in cultured embryonic fibroblasts.

15

The PCR excision assay is used to detect *Minos* excision in cultured embryonic fibroblasts carrying the MCG transgene. Cells are transfected with a plasmid carrying the *Minos* transposase cDNA under CMV control (pJGD/ILMi, figure 1) and analysed by the PCR excision assay. Excision products are detectable in transfected but not in non-
20 transfected cells (data not shown). This result suggests that the transposon transgene is accessible to the *Minos* transposase in tissues other than T cells.

Example 3: Detection of excision events.

25 To determine the nature of the excision events, PCR products from thymus and spleen of MCG/+ TM2/+ mice and from pJGD/ILMi transfected embryonic fibroblasts are cloned and sequenced. The sequence left behind after *Minos* excision in *Drosophila* consists of the TA dinucleotide duplication that is created upon *Minos* insertion, flanking the terminal 4 nucleotides of the transposon (i.e. either a AcgagT or a ActcgT insertion in the
30 TA target site). In the mouse excisions analysed, the size and sequence of the footprints varies considerably (figure 4). Only 2 of the 32 footprints have the typical 6 bp sequence; the others contain extra nucleotides, in addition to complete or partial versions of the typical footprint. Four events have 1-2 nucleotides of the flanking *D. hydei* chromosomal

sequence deleted. The differences in footprint structures observed between *Drosophila* and mouse may reflect the involvement of host factors in *Minos* excision and/or chromatid repair following excision.

5 **Example 4: Detection of transposition in transgenic mice using FISH**

Detection of transposase-dependent excision in thymus and spleen suggests that transposition may also take place in these tissues. The detection of transposition events is not straightforward, because every transposition event is unique, and as a result the tissue in which transposition has occurred will be a mosaic of cells with unique transpositions. Indeed, Southern analysis did not show transposition events in the thymus of double transgenics, indicating that, if such mosaics exists, they consist of small numbers of clonally related cells.

15 Therefore, FISH in metaphase nuclei from the thymus and spleen to detect individual transposition events. A GFP fragment is used as a probe to detect relocation of transposons into new chromosomal positions. The initial position of the array of transposons is at the tip of chromosome 14, at a position indistinguishable from the telomere, as shown by co-localization, in metaphase and interphase chromosomes, with a probe specific for telomeric sequences of chromosome 14 (figure 5, A-B). A total of 20 3,114 metaphases from 5 MCG/+ TM2/+ mice are analysed; 1,688 are from spleen and 1,426 from thymus. Nineteen of these metaphases (11 from spleen and 8 from thymus) show transposition. In addition to the signal at the tip of chromosome 14, pairs of dots are present in these metaphases on chromosomes other than 14, or on a new position on 25 chromosome 14. Representative metaphases are shown in figure 5 (C-F). Morphological analysis of the chromosomes carrying new insertions show that all events except one are independent from each other, *i.e.* they represent different transpositions. Analysis of the positive metaphases with a probe specific for the telomere of chromosome 14 indicates that transpositions do not involve translocation of telomeric material (data not shown). As 30 controls, 2,440 metaphases from thymus and spleens of five MCG/+ mice are screened; no transpositions are detectable in those samples.

This is the first demonstration that a transposase expressed from a transgene can mobilize a transposon to jump into new chromosomal sites in mammalian tissues.

5 All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications
10 of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

Claims

1. A method for generating a transgenic organism, comprising the steps of:
 - 5 (a) providing a first transgenic organism, which organism comprises, within at least a portion of its tissues or cells, one or more copies of a Minos transposon;
 - (b) providing a second organism, which organism comprises, in the genome of at least a portion of its tissues or cells, a Minos transposase or one or more copies of a gene encoding a Minos transposase; and
 - 10 (c) crossing the organism so as to obtain transgenic progeny which comprise, in at least a portion of their tissues or cells, both the Minos transposon and the Minos transposase.
2. A method according to claim 1, wherein the organism is a mammal.
- 15 3. A method for generating a transgenic organism, comprising the steps of:
 - (a) providing a first transgenic organism, which organism comprises, within at least a portion of its tissues or cells, one or more copies of a transposon;
 - (b) providing a second organism, which organism comprises, in the genome of at least a portion of its tissues or cells, a transposase or one or more copies of a gene encoding a transposase; and
 - 20 (c) crossing the organism so as to obtain transgenic progeny which comprise, in at least a portion of their tissues or cells, both the transposon and the transposase.
- 25 4. A method according to any preceding claim, wherein the transposon has been modified to include a heterologous nucleic acid sequence, flanked by inverted terminal repeats homologous to the transposon.
5. A method according to any preceding claim, wherein the transposon has inserted
30 therein a highly-transcribed gene such that, upon integration, the transposon lies in open chromatin structure.

6. A method according to any preceding claim, wherein the transposon is inserted into a highly expressed gene.
7. A method according to claim 6 wherein the transposon is inserted by recombination in ES cells.
8. A method according to claim 3, wherein the transposon is selected from the group consisting of Minos, mariner, Hermes and piggyBac.
9. A method according to claim 8, wherein the transposon is Minos.
10. A method according to any one of claims 4 to 9, wherein the transposon comprises a nucleic acid sequence encoding a selectable marker.
11. A method according to claim 10, wherein the selectable marker is a fluorescent or luminescent polypeptide.
12. A method according to any preceding claim, wherein the transposase has been modified to optimise codon usage.
13. A method according to any preceding claim, wherein the transposase is expressed under the control of control sequences which permit regulation of the expression of the transposase.
14. A method according to claim 13, wherein the control sequences are selected from inducible expression systems including tetracycline, oestrogen and ecdysone inducible systems.
15. A method according to claim 13, wherein the promoter is regulated in response to tissue-specific signals, developmental signals or exogenous signals.
16. A method according to any one of claims 13 to 15, wherein the control sequences comprise a locus control region.

17. A method according to any one of claims 13 to 16 wherein the regulation of expression of the transposase induces excision of a transposon.
- 5 18. A method according to claim 17 wherein excision of the transposon is used to genetically manipulate an organism.
19. A method according to claims 17 or 18 wherein the genetic manipulation of an organism includes the reversion of gene disruptions.
- 10 20. A method for detecting and characterising a genetic mutation in a transgenic organism, comprising the steps of:
- (a) generating a transgenic organism by a procedure according to any preceding claim;
 - 15 (b) characterising the phenotype of the transgenic organism;
 - (c) detecting the position of one or more transposon insertion events in the genome of the organism; and
 - (d) correlating the position of the insertion events with the observed phenotype, the position of the insertion events being indicative of the location of one or
 - 20 more gene loci connected with the observed phenotype.
21. A method for isolating a gene which is correlated with a phenotypic characteristic in a transgenic animal, comprising the steps of:
- 25 (a) generating a transgenic organism by a procedure according to any one of claims 1 to 19;
 - (b) characterising the phenotype of the transgenic organism;
 - (c) detecting the position of one or more transposon insertion events in the genome of the organism; and
 - (d) cloning the genetic loci comprising the insertions.
- 30 22. A method for isolating an enhancer in a transgenic animal, comprising the steps of:

- (a) generating a transgenic organism by a procedure according to any one of claims 1 to 19, wherein the transposon comprises a reporter gene under the control of a minimal promoter such that it is expressed at a basal level;
- (b) assessing the level of expression of the reporter gene in one or more tissues of the transgenic organism;
- (c) identifying and cloning genetic loci in which the modulation of the reporter gene is increased or decreased compared to the basal expression level; and
- (d) characterising the cloned genetic loci.
23. A method for isolating an enhancer in a transgenic animal, comprising the steps of:
- (a) generating a transgenic organism by a procedure according to any one of claims 1 to 19, wherein the transposon comprises a reporter gene which lack translation initiation sequences but includes splice acceptor sequences;
- (b) identifying tissues of the organism in which the reporter gene is expressed;
- (c) cloning the genetic loci comprising the expressed reporter gene.
24. A method for isolating an exon in a transgenic animal, comprising the steps of:
- (a) generating a transgenic organism by a procedure according to any one of claims 1 to 19, wherein the transposon comprises a reporter gene under the control of a minimal promoter such that it is expressed at a basal level;
- (b) assessing the level of expression of the reporter gene in one or more tissues of the transgenic organism;
- (c) identifying and cloning genetic loci in which the modulation of the reporter gene is increased or decreased compared to the basal expression level; and
- (d) characterising the cloned genetic loci.
25. A method for isolating an exon in a transgenic animal, comprising the steps of:
- (a) generating a transgenic organism by a procedure according to any one of claims 1 to 19, wherein the transposon comprises a reporter gene which lack translation initiation sequences but includes splice acceptor sequences;
- (b) identifying tissues of the organism in which the reporter gene is expressed;
- (c) cloning the genetic loci comprising the expressed reporter gene.

26. A method for modulating the expression of a gene in an organism, comprising the steps of:
- 5 (a) generating a library of transgenic organisms according to any one of claims 1 to 19;
- (b) selecting from said library one or more transgenic organisms in which the expression of a gene of interest is modulated as a result of one or more transposon insertion events.
- 10 27. A transgenic organism comprising one or more copies of a heterologous transposon, said transgenic organism being free of nucleic acid sequences encoding the cognate transposase enzyme.
- 15 28. A transgenic organism encoding a transposase enzyme, said transgenic organism being free of the cognate transposon.
29. An organism according to claim 27 or claim 28, which is a mammal or a plant.
- 20

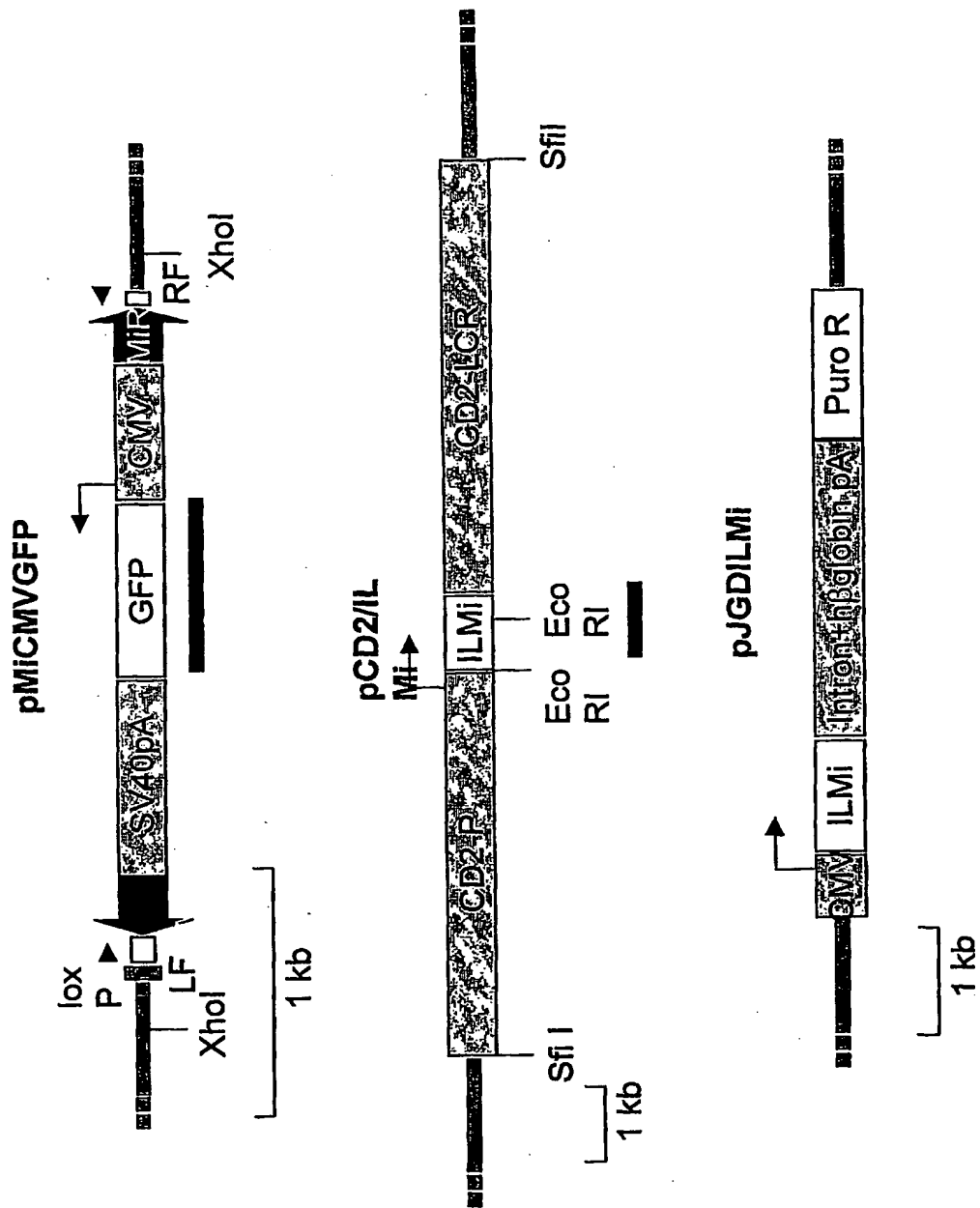


Figure 1

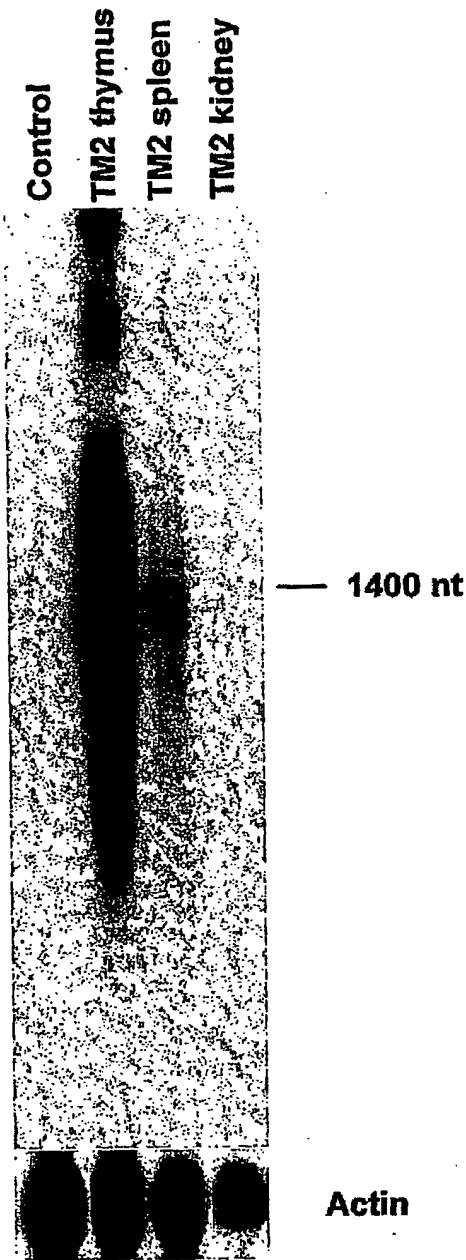


Figure 2

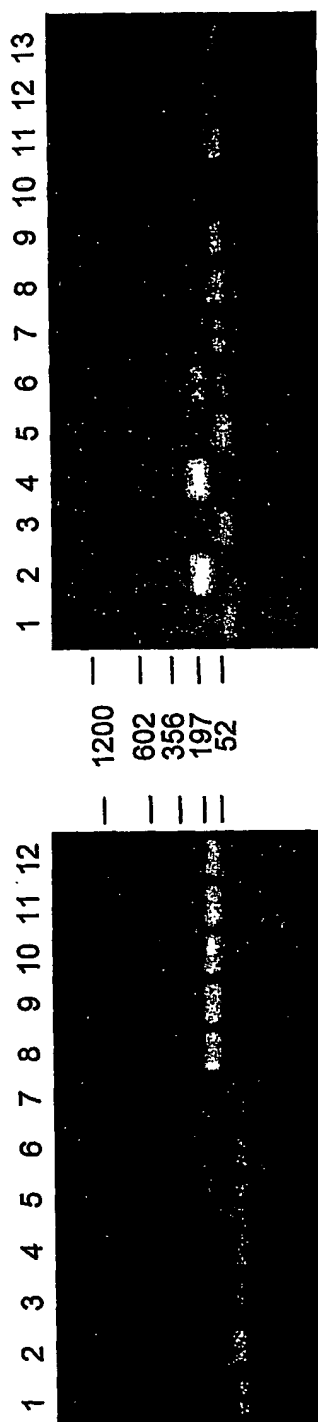


Figure 3

Thymus and spleen

...ttc ctcgTAgaa...
 ...ttcT cgTAgaa...
 ...ttcTA ctcgTAgaa...
 ...ttcTAc cgTAgaa...
 ...ttcTAc ctcgTAgaa...
 ...tccTAcg TAgaa...
 ...ttcTAcg TAgaa...
 ...ttcTAcgag TAgaa...
 ...ttcTAcgag gTAgaa...
 ...ttcTAcgagg gTAgaa...
 ...ttcTAcgagg cgTAgaa...
 ...tccTAcgagaa TAgaa...
 ...tccTAcgagaa TAgaa...
 ...ttcTAcgagag gTAgaa...
 ...ttcTAcgaggg gTAgaa...
 ...ttcTAcgaggg gTAgaa...
 ...ttcTAcgagtctcgTAgaa...
 ...ttcTAcgagact gTAgaa...
 ...ttcTAcgagttggggTAgaa...

Embryonic fibroblasts

...t cgTAgaa...
 ...t cgTAgaa...
 ...ttcT cgTAgaa...
 ...ttcT cgTAgaa...
 ...ttcT cgTAgaa...
 ...ttcT cgTAgaa...
 ...ttcT cgg Agaa...
 ...ttcTA aa...
 ...ttcTAc TAgaa...
 ...ttcTAc aa...
 ...ttcTAg gTAgaa...
 ...ttcTAggag gaa...
 ...ttcTAgtag gaa...

Figure 4

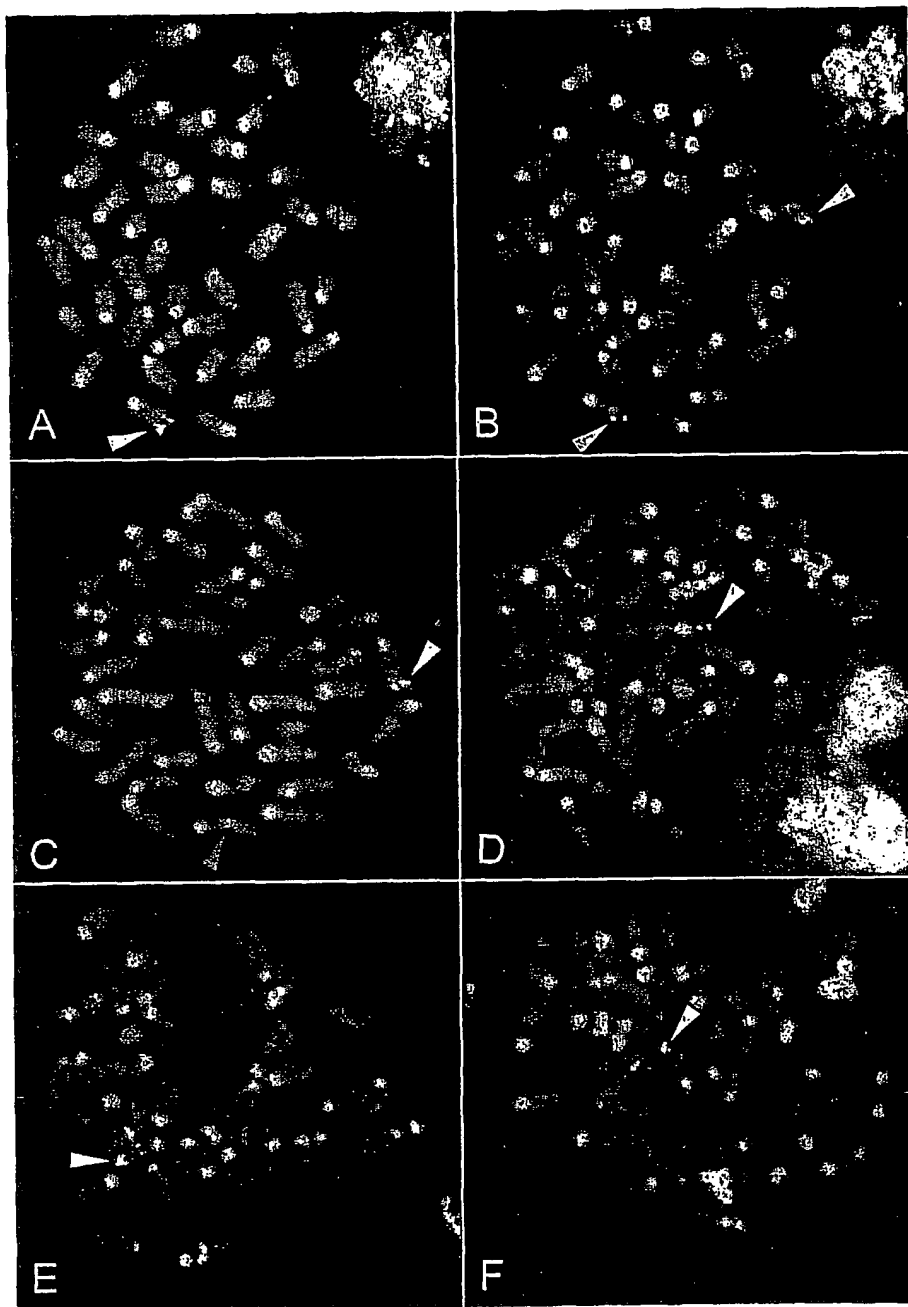


Figure 5

INTERNATIONAL SEARCH REPORT

International Application No

EP 01/03341

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12N15/90 A01K67/027 A01K67/033 A01H1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C12N A01K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 01370 A (UNIV CALIFORNIA) 6 February 1992 (1992-02-06) page 7, line 17 - page 8, line 16 page 29, line 5 - line 15 page 30, line 47 - line 50 page 32, line 21 - line 35; claims 1,2,13	3-7, 10-29
Y	— -/-	1,2,8,9

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

26 July 2001

Date of mailing of the international search report

17/08/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+31-70) 340-3018

Authorized officer

van Heusden, M

INTERNATIONAL SEARCH REPORT

Int. Application No.

P 01/03341

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HONMA MARY A ET AL: "High-frequency germinal transposition of Ds-ALS in Arabidopsis." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 90, no. 13, 1993, pages 6242-6246, XP002172889 1993 ISSN: 0027-8424 page 6242; figure 1	3-7, 10-29
Y		1,2,8,9
X	COOLEY L ET AL.: "Insertional mutagenesis of the Drosophila genome with single P elements" SCIENCE, vol. 239, 1988, pages 1121-1128, XP001008878 page 1122, right-hand column, paragraph 2; figure 1	3-7, 10-29
Y		1,2,8,9
X	EP 0 955 364 A (INST MOLECULAR BIOLOGY AND BIO) 10 November 1999 (1999-11-10) page 17, line 3 - line 16	28,29
Y		1,2,8,9
Y	US 5 614 398 A (ATKINSON PETER ET AL) 25 March 1997 (1997-03-25) abstract	8
X	WO 99 09817 A (FINNEGAN DAVID JOHN ;SANG HELEN (GB); BIOTECH & BIOLOG SCIEN RES ()) 4 March 1999 (1999-03-04) page 3, line 13 - line 17 page 4, line 11 - line 21 figure 1; examples 2,3; table 1	27,29
Y		8
Y	LOUKERIS T G ET AL: "Introduction of the transposable element Minos into the germline of Drosophila melanogaster" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,NATIONAL ACADEMY OF SCIENCE. WASHINGTON,US, vol. 92, October 1995 (1995-10), pages 9485-9489, XP002163150 ISSN: 0027-8424 the whole document	1,2,8,9

-/-

INTERNATIONAL SEARCH REPORT

International Application No.

P 01/03341

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HANDLER AM, HARRELL RA: "Germline transformation of Drosophila melanogaster with the piggyBac transposon vector" INSECT MOL. BIOL., vol. 8, no. 4, 1999, pages 449-457, XP002172890 the whole document	8
A	KLINAKIS AG ET AL.: "Genome-wide insertional mutagenesis in human cells by the Drosophila mobile element Minos" EMBO REPORTS, vol. 1, no. 5, 2000, pages 416-421, XP002172891 the whole document	1-29
P,A	KLINAKIS A G ET AL: "Mobility assays confirm the broad host-range activity of the Minos transposable element and validate new transformation tools" INSECT MOLECULAR BIOLOGY, BLACKWELL SCIENTIFIC, OXFORD,, GB, vol. 9, no. 3, June 2000 (2000-06), pages 269-275, XP002163113 ISSN: 0962-1075 the whole document	1-29
P,A	WO 00 17343 A (WISCONSIN ALUMNI RES FOUND) 30 March 2000 (2000-03-30) the whole document	20-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent Application No

PCT/EP 01/03341

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9201370	A	06-02-1992	US 5225341 A	06-07-1993
			AU 660620 B	06-07-1995
			AU 8102291 A	18-02-1992
			EP 0577598 A	12-01-1994
			JP 2659459 B	30-09-1997
			JP 5508993 T	16-12-1993
			US 5482852 A	09-01-1996
			US 5792924 A	11-08-1998
EP 0955364	A	10-11-1999	US 6225121 B	01-05-2001
US 5614398	A	25-03-1997	NONE	
WO 9909817	A	04-03-1999	AU 8817798 A	16-03-1999
			EP 1006790 A	14-06-2000
WO 0017343	A	30-03-2000	US 6159736 A	12-12-2000
			AU 6057399 A	10-04-2000
			EP 1115856 A	18-07-2001

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.